# Stratification of Living Organisms in Ballast Tanks: How Do Organism Concentrations Vary as Ballast Water Is Discharged?

Matthew R. First,[*,†] Stephanie H. Robbins-Wamsley,[†] Scott C. Riley,[†] Cameron S. Moser,[‡] George E. Smith,[§] Mario N. Tamburri,[⊥] and Lisa A. Drake[¶]

[†]SAIC, Inc., Key West, Florida 33041, United States

[‡]EXCET, Inc., Key West, Florida 33041, United States

[§]Smithsonian Environmental Research Center, Edgewater, Maryland 21037, United States

[⊥]Chesapeake Biological Laboratory, University of Maryland Center for Environmental Science, Solomons, Maryland 20688, United States

[¶]Chemistry Division, Naval Research Laboratory, Code 6136, Key West, Florida 33041, United States

**ABSTRACT:** Vertical migrations of living organisms and settling of particle-attached organisms lead to uneven distributions of biota at different depths in the water column. In ballast tanks, heterogeneity could lead to different population estimates depending on the portion of the discharge sampled. For example, concentrations of organisms exceeding a discharge standard may not be detected if sampling occurs during periods of the discharge when concentrations are low. To determine the degree of stratification, water from ballast tanks was sampled at two experimental facilities as the tanks were drained after water was held for 1 or 5 days. Living organisms $\geq 50 \mu$m were counted in discrete segments of the drain (e.g., the first 20 min of the drain operation, the second 20 min interval, etc.), thus representing different strata in the tank. In 1 and 5 day trials at both facilities, concentrations of organisms varied among drain segments, and the patterns of stratification varied among replicate trials. From numerical simulations, the optimal sampling strategy for stratified tanks is to collect multiple time-integrated samples spaced relatively evenly throughout the discharge event.
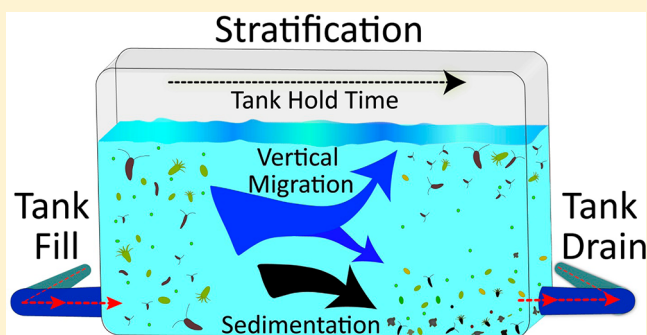
## INTRODUCTION

Ships use ballast water to maintain stability and manage draft and trim. However, ballast water can also contribute to the global dispersal of aquatic invasive species.[1] Establishment of invasive species can have profound impacts on the environment, including major shifts in food webs,[2] displacement of native species,[3] and potential consequences to human health[4] and infrastructure.[5] To minimize the introduction of invasive species through ballast water, limits have been established for the maximum concentrations of living organisms allowable in discharged water.[6,7] Ballast water management systems (BMWSs), which are designed to kill or remove aquatic organisms, are currently being developed and employed to ensure discharge water complies with national and international discharge standards.[8]

Discharge standards ensconced in the International Convention on the Control and Management of Ships' Ballast Water and Sediments[6] and promulgated by the U.S. Coast Guard (USCG) are based upon the size of organisms. For example, <10 organisms in the largest size class ($\geq 50 \mu$m in minimum dimension, dominated by zooplankton) are permitted per m[3] of discharged water.[6,9] To provide statistical confidence to estimates of these sparse populations, the volume of sample water collected to enumerate organisms in this size class must be representative of the volume of interest (e.g., whether an entire ballast tank or 1 m[3] of ballast water).[10,11] Samples could be collected either: (1) as a single sample, collected throughout the entire discharge event or (2) as a single or multiple samples from a portion of the entire discharge event. Regardless of the sampling strategy used, to ensure that samples are representative of the entire volume of interest, samples must be collected in a time-averaged manner and imparting minimum mortality to organisms, e.g., using L-shaped, isokinetic sampling ports.[7] In the field experiments described here, all samples were collected as such.

Stratification due to vertical migrations of zooplankton, patchiness of organisms taken up in source water, accumulation of plankton near walls and surfaces within ballast tanks, avoidance of pumps, and the settling of organisms results in uneven distributions of organisms in ballast water tanks. Such redistribution throughout the ballast tank is likely most

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pronounced for large (i.e., ≥50 $\mu$m) aquatic organisms, a group that is generally motile and includes microinvertebrates, animal larvae, heterotrophic protists, and some microalgae (e.g., diatoms). Indeed, certain planktonic organisms such as copepods are known to undergo daily, extensive (>10 m) vertical migrations.[12] Diatoms can regulate cell buoyancy, which permits vertical redistribution in response to light and nutrient gradients.[13] Organisms can also be redistributed due to particle aggregation and settling, which enriches deeper strata with particle-associated organisms.[14,15]

The stratification of organisms in a ship's ballast tank has been investigated in a prior study, which found that certain organism groups displayed uneven distributions throughout the depth of the tank and that these distribution patterns varied over time.[16] While providing important real-world evidence of the uneven distribution of plankton in ballast tanks, the Murphy et al. study was conducted on two voyages of one vessel, and samples were collected by pumping water from three distinct depths that did not sample the entire vertical distribution of the ballast tank. For BWMS verification testing and future compliance monitoring of ballast discharges, samples must be collected in a time-averaged manner and from inline sample ports, as water is being discharged.[6,7]

This study determined concentrations and distributions of living aquatic organisms ≥50 $\mu$m in ballast tanks located at two test facilities with different ambient water (brackish or marine). Continuous, time-integrated samples were collected from an inline sample port located in the drain line of the tank. Water was held for 1 or 5 days (d) prior to draining, and the entire discharged volume from the ballast tank (200−300 m$^3$) was sampled in a time-averaged manner as it was emptied, but importantly, multiple sampling devices were used throughout sampling so the concentration of organisms could be determined over different intervals or segments. For example, three mesh nets were used sequentially to collect samples from three drain segments of the discharge event (beginning, middle, and end). Concentrations of living organisms ≥50 $\mu$m were measured in each of the drain segments to test the hypotheses that (1) concentrations of living organisms vary throughout the course of a tank's discharge and (2) these variations could significantly affect the estimate of organism concentration in the volume of interest (here, the entire ballast tank) if samples were taken from any one drain segment alone. Understanding the magnitude of organism stratification in ballast tanks upon discharge (both from experiments such as these and data collected during shipboard validation trials) will allow for the development of optimal sampling strategies, which will minimize sampling effort and yield accurate estimates of the entire volume of interest.

## ■ EXPERIMENTAL SECTION

Experiments were conducted at the Naval Research Laboratory in Key West, Florida (NRL) and the Maritime Environmental Resource Center in Baltimore, Maryland (MERC). Both facilities have conducted large-scale (200 to 300 m$^3$) experiments testing BWMS using ambient water. However, there were several notable differences between the facilities regarding water characteristics, sampling methodologies, and approaches to counting living organisms. NRL employed an above ground, model ballast tank (∼225 m$^3$ volume) and performed tests by amending the ambient marine (salinity: 33−38 psu), oligo-trophic water with mineral and organic matter.[17] The model ballast tank used at NRL was rectangular, with structural support beams on the interior. MERC performed tests using 650 m$^3$

ballast tanks (filled to ∼300 m$^3$) on the M/V *Cape Washington* (US Maritime Administration ready reserve, Roll On-Roll Off vessel) while dockside in the Port of Baltimore, which is situated in the brackish (salinity: 8−12 psu), eutrophic waters of the Chesapeake Bay. The ballast tanks of the M/V *Cape Washington* were filled to a height of 2 m, and at this depth, the tanks were rectangular with one sloping edge and structural support beams on the interior. Other differences in experimental design and sample analysis, when relevant, are described below. At both facilities, testing was conducted using the tank volumes, sample volumes, and analysis methods described in the Environmental Technology Verification (ETV) Protocol for verification of BWMS,[7] which was developed by the EPA and USCG with the input of technical experts and stakeholders to verify the performance of BWMSs.

**Experimental Design.** *NRL.* Experiments were conducted at NRL during Feb−Apr 2011 by filling a model ballast tank with 200 m$^3$ of ambient water and holding the water for either 1 or 5 d prior to discharge and sampling. Three replicate trials were performed for each hold time, and water was amended to meet the minimum concentrations of dissolved organic carbon (DOC), particulate organic matter (POM), and mineral matter (MM) as specified in the ETV Protocol.[7] After 1 or 5 d, water was drained through the seawater piping at approximately 230 m$^3$ h$^{-1}$. Time-integrated samples were collected from the main drain line as described elsewhere.[7] Sampled water (approximately 5% of the total volume of water in the tank) was conveyed through a flexible hose into a filter skid, which is a sampling device composed of two stainless steel filter housings, each with filter bag made of monofilament mesh (35 $\mu$m; 50 $\mu$m diagonal length) netting (81 cm long; 18 cm mouth diameter).[18] The water from the ballast tank was sampled through the filter skid until approximately one-third of the tank was drained (approximately 20 min). At this point, the sample flow was switched to a second filter skid, which had the same specifications as the first. Rerouting the flow between different arrays of the filter skid required manually turning two valves, which took <30 s. While the middle portion (i.e., the second of three segments) of the drain was sampled, the filter bags from the first segment sampling were removed for processing, and new filter bags were placed in the housings. After the second drain segment was completed, the sample flow was routed back to the first array of the filter skid. Each of the three sample segments collected a time-integrated sample from, on average, 33% (range: 26−38%, n = 18) of the total tank volume. The average of the total water volume sampled from the 200 m$^3$ volume in the tank (i.e., the sum of all three segments) was 9.8 m$^3$ (range: 8.3 to 12 m$^3$, n = 6). The first, second, and third samples collected represented approximately the bottom third, the middle third, and the top third of the tank, respectively. Each segment corresponded to approximately 1.1 m in depth; thus, the second and third segments represented water columns of approximately 2.2 and 3.3 m high.

To determine the difference between the concentration of organisms entering the tank versus leaving the tank after 1 or 5 d, samples for living and dead organisms were collected as the tank was filled. A time-integrated sample (3.8 ± 0.3 m$^3$, mean ± 1 SD; range: 3.5−4.3 m$^3$, n = 6) was collected during the entire time it took to fill the tank (∼1 h). Drain concentrations were normalized to fill concentrations to demonstrate the mean change in organisms over the tank hold time (described below in the Data Analysis section).

*MERC.* Experiments at MERC were conducted from Mar–Aug 2009 by filling two identical ballast tanks with approximately 300 m³ of ambient water and holding water for 5 d (see www.maritime-enviro.org/reports.php for more details). The water in one of these tanks was treated by a BWMS to inactivate organisms; only the results from the untreated water (i.e., in the control tank) for three replicate trials are reported here. Samples at MERC were collected in a time-averaged manner and sequentially into 1 m³ sample tanks throughout the tank drain operation. Between four and five separate 1 m³ sample tanks were filled sequentially over the course of sampling, and they were immediately drained through a 35 µm mesh (50 µm diagonal length) plankton net to concentrate organisms into volumes of approximately 500 mL. As at NRL, the first sample represented the bottom portion of the ballast tank (the bottom fourth or fifth, depending on the number of samples collected); subsequent samples represented strata increasingly higher in the ballast tank. Each drain segment corresponded to approximately 0.4–0.5 m in depth; thus, the second segment represented a water depth of approximately 0.8–1.0 m depth, etc. These samples were processed and analyzed separately, and the data from the segments were averaged to calculate the overall tank concentration.

**Sample Processing and Analysis.** Detailed sample processing and analysis protocols used at both facilities are available elsewhere;[7,18] a short description of the methods used at each facility follows.

*NRL.* Briefly, material collected in the filter skid during a given drain segment was rinsed and concentrated to a final volume of 0.8 to 1.0 L. Filtered seawater (FSW) was prepared by filtering ambient seawater through fine glass fiber filters (GF/F; Whatman, Inc.; Piscataway, NJ), which effectively removes particulates >0.7 µm. The FSW, which was prepared within 48 h prior to the test, was used to rinse the filter bags and to dilute the concentrated samples. All items in contact with the concentrated sample (e.g., glassware, sieves, etc.) were well rinsed with FSW to minimize loss of collected material. Sample processing began upon completion of each drain segment, and processing time for each set of filter bags (i.e., a drain segment) was generally <20 min. All analyses were complete within 6 h of sample collection.

Five subsamples (4 mL each) were removed from each sample. These analytical replicates provided an estimate of the analysis error, which is reflective of both sampling and counting procedures. A study describing the counting procedures and supporting quality assurance quality control measures is described elsewhere.[17] Briefly, samples were homogenized by gently inverting them 3× and removing five analytical replicates with a pipet. Subsamples were transferred into Bogorov chambers, which are acrylic plates with a single sinuous chamber, and examined at 30× magnification. Polystyrene microbeads with a known diameter (49 ± 1.5 µm, Chromosphere; Fisher Scientific, Pittsburgh, PA) were added to the Bogorov chambers to provide a size reference. All organisms (both living and dead) larger than the microbeads were counted. If they were motile, organisms were tallied as "living". Nonmoving organisms were gently touched using a small metal probe, and if this action stimulated movement within 10 s, the organism was determined to be living;[7] if not, the organism was tallied as "dead". Some organisms (e.g., diatoms) would not be expected to move in response to this stimulus. Therefore, these organisms were classified as living if the cellular structures (e.g., chloroplasts and cell walls) were intact.

A portion of the concentrated sample collected from each of the drain segments at NRL was analyzed to determine the concentration of total suspended solids (TSS) by filtering a known volume of water through a glass fiber filter (GF/F), rinsing with deionized water to remove salts, drying to a constant mass at 104 °C, and weighing the dried material. The material examined was retained in filter bags with 35 µm mesh netting, which effectively retains particles >50 µm. The concentration of TSS is indicative of degree of particle stratification in the tank: relatively high concentrations of TSS in the bottom tank strata (the first drain segment) indicate high rates of particle aggregation and settling. Tracking TSS in different drain segments also provides a comparison between replicate trials. In real-world situations, such as aboard ships, TSS distribution may indicate the degree of tank mixing.

*MERC.* The proportion and total concentration of living versus dead organisms was determined using standard movement and response to stimuli techniques, consistent with the ETV protocol,[7] and analysis occurred within 1 h of collecting the individual samples. Total counts were conducted under a dissecting microscope at 25× magnification, except for some taxa, which were removed and identified using a compound microscope at higher magnification. All analyses were complete within 2 h of collection.

*Data Analysis.* The counts of organisms were converted into concentrations ($P$, ind. m$^{-3}$) using the volume of sample ($S$, m³), the volume of concentrated sample ($C$, mL), the aliquot volume that was analyzed ($A$, mL), the dilution ($D$, e.g., 10 for a 1:10 or 10× dilution), and the number of individuals counted in the aliquot ($I$).

$$P = \frac{ICD}{SA} \tag{1}$$

This equation was used to calculate total concentration of both living and dead aquatic organisms ≥50 µm. Results of replicate trials are shown individually, and statistical comparisons (pairwise $t$ tests, $\alpha = 0.05$) were based upon subsamples of a single sample (i.e., analytical replicates). However, each sample represents a time-averaged segment of the drain, and treating the analytical replicates as independent estimates of concentrations in the time average sample allowed for comparisons among drain segments within a single drain event. To examine stratification among multiple trials, organism concentrations in drain samples at NRL were normalized to fill concentrations to demonstrate the relative change in living or dead organism concentrations over the tank hold time. This normalization allows multiple trials (each with a different starting concentration of organisms) to be combined and averaged. Error propagation formulas were used to determine the total error of values obtained from calculations consisting of multiple measurements, each with an associated error.[19]

**Simulated Sampling of Living Organisms.** To further investigate the optimal strategy for sampling heterogeneous distributions of organisms in ballast tanks, two types of organism distributions were simulated, both representing ballast tanks with a total concentration of 10 organisms m$^{-3}$ (Matlab, V7.12; Mathworks, Natick, MA): a bimodal distribution, where high concentrations of living organisms are found at the bottom and the top of the tank, and a unimodal distribution, where living organisms accumulate at the top of the tank and thus are captured at the end of the discharge. Next, sampling strategies were simulated in which different sample volumes (3, 5, 7, and 10 m³) were collected in increments of 1 m³. Representative samples

of the drain were collected either randomly or spaced evenly throughout the drain. For example, an evenly spaced sample of 5 m³ would collect 1 m³ from each of five segments at equal-spaced intervals throughout the drain (within the segment, the sample was collected randomly). Following these constraints, the distributions of organisms were sampled 1000 times and the mean concentration of organisms (org. m⁻³) was calculated for the total volume sampled. For simplicity, no error was attributed to the sampling or counting procedures, and sample flow was considered to be uniform throughout the discharge and sampled in a representative manner. This procedure indicates the range of concentration estimates that are possible when subsampling a distribution with a known concentration.

## ■ RESULTS AND DISCUSSION

**Stratification of Organisms in Ballast Tanks.** Both 1 and 5 d hold trials were performed at NRL. For all 1 d trials, concentrations of living organisms ≥50 μm in the final drain segment were significantly higher than the first segment (Figure 1). In trials 1 and 3, the concentration of living organisms in the final segment was significantly higher than the previous two segments, and in trial 2, the concentration in the final segment was significantly higher than the first segment but not the second
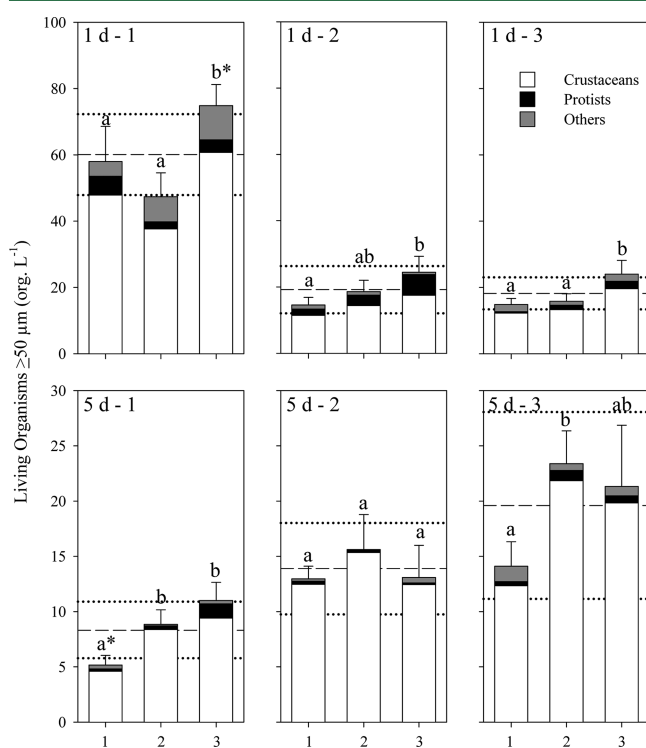


**Figure 1.** Concentrations of living organisms ≥50 μm (org. L⁻¹) in different segments of tank drain from trials conducted at the Naval Research Laboratory (NRL). The bars show the mean concentration (±1 SD) of five analytical replicate readings from a single sample, and the relative abundance of crustaceans, protists, and other organisms are shown as partitions within the bars. Significantly different concentrations among segments (t test, p < 0.05) are marked by different letters, and an asterisk indicates the organism concentration in the drain segment is significantly different from the grand mean. The solid and dotted lines show the grand mean concentration and values 1 SD from the mean, respectively (n = 3 segments). The panels are identified by tank hold time (1 or 5 d) and the trial number (1, 2, or 3). Note the differences in the y-axes.

(Figure 1). In trials with a 5 d hold time, trial 1 displayed a similar trend and had the highest concentrations in the final drain segment (Figure 1). Concentrations of organisms were significantly lower in the first drain segment than the second segment in trial 3 of the 5 d tank hold. In contrast, differences in organism concentrations were not significant in trial 2 of the 5 d hold trials (Figure 1).

At NRL, the concentration of organisms in a segment was significantly different from the grand mean (i.e., the average of all segments) in only two instances (1 d hold time, trial 1 and 5 d hold time, trial 1). Differences in drain segment concentrations, when normalized to fill concentrations and averaged, were not significantly different (Figure 2). Concentrations of living
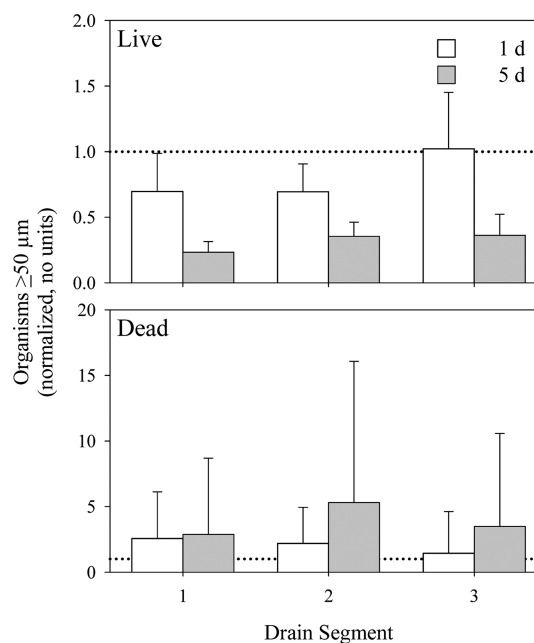


**Figure 2.** Normalized concentrations of living (top panel) and dead (bottom panel) organisms in the drain segments in both 1 and 5 d trials at NRL. The bars show normalized mean (±1 SD) of three replicate trials. The dotted lines mark equal concentrations in fill and drain samples; values below this threshold show a loss over the hold time, whereas values above this line show an increase during the hold time. Significant differences between normalized concentrations in segments were not observed. Note the differences in the y-axes.

organisms decreased throughout the tank hold time, and this decrease occurred in tandem with an increase in the concentration of dead organisms. This pattern was most evident in 5 d tank hold trials (Figure 2).

At MERC, the abundance of living organisms varied among the discharge segments. However, the pattern of abundance was not consistent among the three replicate trials. In two of three trials, the first drain segment had significantly higher living organism concentrations than the subsequent drain segments (Figure 3). In the other trial, organism concentrations in the final drain segment were significantly higher than two of the previous drain segments (t test, p < 0.05). The concentrations of organisms in drain segments, in several cases, were significantly different from the grand mean concentration (i.e., the average of all the segments, e.g., Figure 3).

The majority of organisms encountered in all NRL discharge segment samples were crustaceans, which were dominated by copepod nauplii and adult copepods (86 ± 8% of the total living
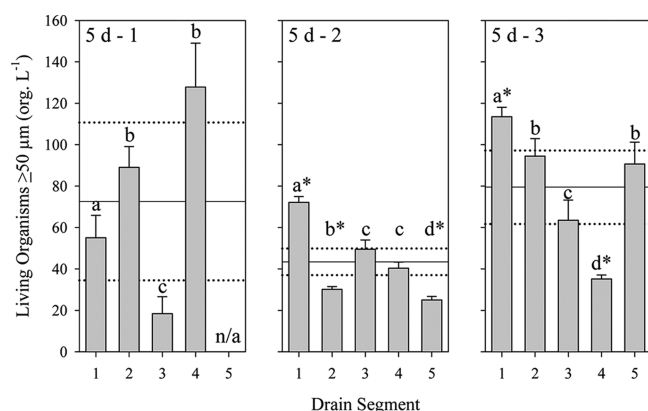
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**Figure 3.** Concentrations of living organisms ≥50 μm (org. L$^{-1}$) in different segments of tank drain samples from trials conducted at the Maritime Environmental Resource Center (MERC). Ambient water had been held for 5 d prior to discharge and sampling. The bars show the mean concentration (±1 SD) of three analytical replicates from a single sample. Significantly different concentrations among segments (*t* test, *p* < 0.05) are marked by different letters, and an asterisk indicates the organism concentration in the drain segment is significantly different from the grand mean. The solid and dotted lines show the mean concentration and values 1 SD from the mean, respectively (*n* = 5 segments, except in trial 1, where *n* = 4). The panels are identified by the tank hold time and trial number (1, 2, or 3). n/a = data not available.

concentration, mean ±1 SD, *n* = 18; Figure 1). Copepods are capable of migrating large vertical distances in the water column. These vertical migrations patterns have been observed over the course of the day and occur to avoid predation[20] or in response to diel patterns of solar intensity.[12] In a ballast tank, light penetration (e.g., through vents) may be limited or nonexistent. Two small hatches (size <1 m$^2$) were open at the top of the tank at NRL (this feature is not unprecedented, as cargo holds in ballast may have open hatches or vents that allow light into the tanks). The small amount of light penetrating may have been sufficient to drive migrations of organisms ≥50 μm (in this case, copepods) to the top strata of the ballast tank. This occurrence was more pronounced in 1 d trials at NRL, potentially due to endogenous rhythms, which can drive migration patterns even in the apparent absence of stimuli.[21,22] During longer tank hold times, inactive and moribund organisms may settle to deeper strata, which may explain the lower concentrations in the top of the tank (which is sampled at the end of the drain) during 5 d hold trials.

Another major process that drives tank stratification is particle settling. In this case, the concentration of total suspended solids, which includes both biotic and abiotic particles, signifies the cumulative amount of particle settling. In all 1 d trials at NRL, the first drain segment had significantly higher concentrations of ≥50 μm suspended solids than the second drain segment (Figure 4). In Trials 2 and 3 of the 1 d tank hold experiments, the first and last drain segments were not significantly different. The first drain segment contained notably high concentrations of particles ≥50 μm in both Trial 1 of the 1 d hold trials (3.7 ± 0.6 g L$^{-1}$) and Trial 3 of the 5 d tank hold experiments (2.7 ± 0.2 g L$^{-1}$).

The processes generating high particle loads in these trials are not clear; however, particle aggregation is encouraged by the certain microbial processes, including bacterial colonization[23,24] and protist phagotrophy.[25,26] For example, the presence of sessile ciliates on particles can accelerate particle growth.[27] Therefore, fluctuations in the microbial community composition, which
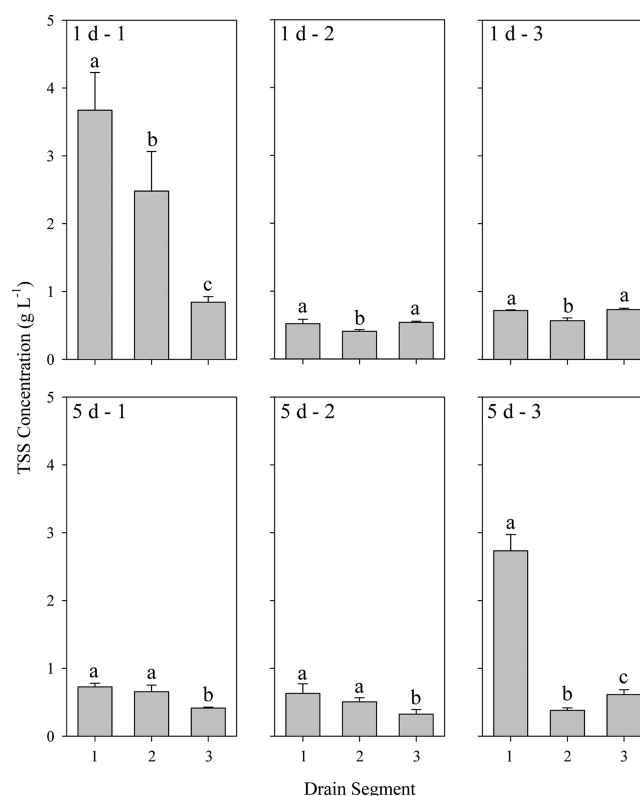


**Figure 4.** Concentration of total suspended solids (TSS; g L$^{-1}$) ≥50 μm in drain segments of 1 d trials (top panels) and 5 d trials (bottom panels) at NRL. Segment 1 was the first sample collected as the tank was drained, followed by segments 2 and 3 (see text). Bars show the mean ±1 SD of analytical replicates (*n* = 3) collected from a single sample. Significantly different concentrations among concentrations (*t* test, *p* < 0.05) are marked by different letters.

could occur over long-term (seasonal) or short-term (tidal) periods, could result in different rates of microbial processes. Furthermore, the addition of MM, DOC, and POC to meet the conditions specified by the ETV Protocol[7] may have contributed to the generation of large particulates (in this case, composed mostly of organic matter). This provision is included in the ETV Protocol to ensure BWMSs are tested under challenging conditions that vessels may encounter; nonetheless, the high organic matter loading in these experiments (and at other land-based test facilities) may fuel the accumulation of large particulates. Subtle changes in the microbial community assemblage (or environmental conditions in the tank) can potentially lead to high rates of particle aggregation. In practice, high particle loading requires longer times to analyze ballast water samples for living organisms, as sample dilution is required to minimize debris that can obscure organisms.[17]

The variations observed between these replicate trials indicate that, even for a single tank filled with water from a fixed location with a similar community composition, the concentrations of living organisms vary in different segments of the discharge, and the patterns of variation were inconsistent among trials. These observations demonstrate vertical distributions of organisms in the tank are not uniform. Subtle variations in the planktonic community, which can occur over short time periods due to tidal advection[28] or food-web interactions,[29] will make the stratification patterns difficult to predict. Therefore, from the data collected in this study, it is not feasible to make a priori predictions of which discharge segments are most representative

of the entire discharge volume or are most likely to contain relatively higher concentrations of organisms.

**Simulated Sampling of Living Organisms.** In an effort to further understand sampling strategies, two simulated organism distributions were created to represent (1) a bimodal distribution, with high concentrations of living organisms at both the bottom and the top of the tank, and (2) a unimodal distribution, with living organisms concentrated at the top of the tank and, therefore, concentrated at the end of the discharge (Figure 5). In terms of minimizing the coefficient of variation
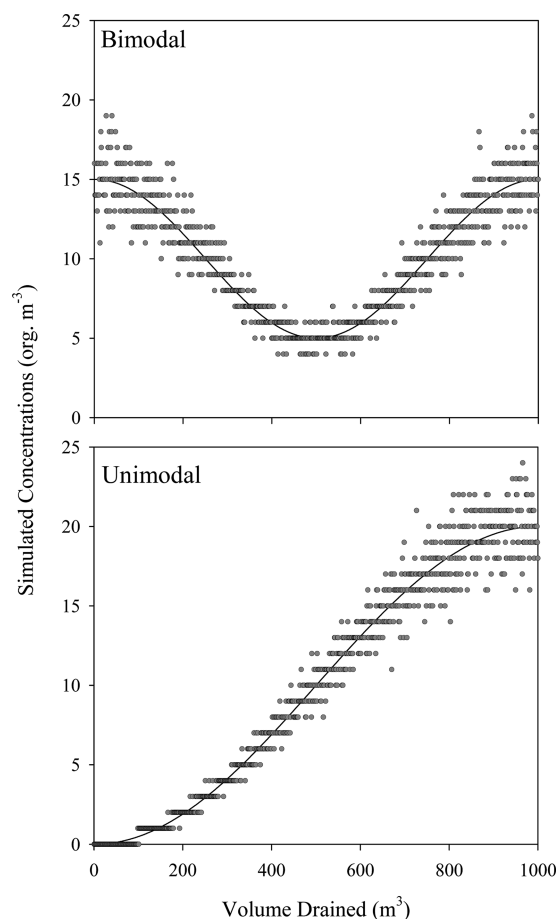


**Figure 5.** Simulated distributions of organisms in ballast water samples. The number of living organisms (org. m$^{-3}$) is randomly generated from either a bimodal (top panel) or unimodal (bottom panel) distribution; in both cases, the final concentration of organisms ≥50 μm is 10 org. m$^{-3}$. Solid circles show the actual concentration found in each m$^3$ of the drain sample, and the solid line shows the function used to generate random concentrations in each m$^3$ of the drain sample.

(CV) and reducing the frequency of over- or underestimates of organisms, the best sampling strategy is to collect samples evenly as the tank is drained (Figure 6). As expected, the largest total sample volumes (10 m$^3$) yielded the lowest CV (Figure 6). However, increasing sample size likely produces "diminishing returns" in lowering CV, and smaller sample sizes (5−7 m$^3$) may be ideal as inline sample devices may produce pressure gradients that lead to organism loss and mortality when large volumes are sampled.[18] While more empirical data are needed to substantiate the findings of this heuristic model, it appears that a sampling strategy where samples are collected evenly throughout the discharge and in a manner that is representative of the water in
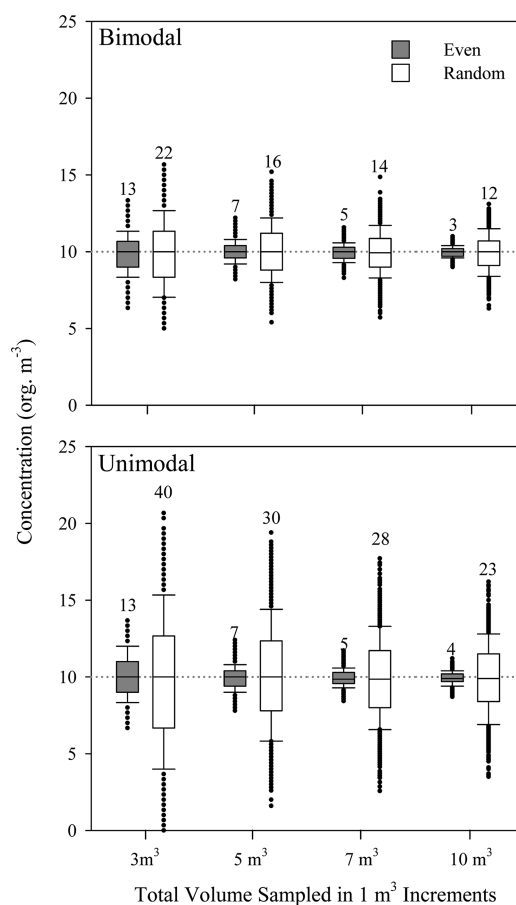


**Figure 6.** Results of sampling the bimodal (top panel) and unimodal (bottom panel) simulated organism distributions (see Figure 5). Sampling can be spaced either randomly or evenly throughout the drain. The box plots represent the distribution of mean organism concentrations from 1000 sampling events. Outer error bars of the box plot show the 10th and 90th percentiles of the data; the bar boundaries show the 25th and 75th percentile values, and the median value is marked by a vertical line within the bar. Extreme values (<10th and >90th percentiles) are marked by black circles. The dotted line indicates the known concentration of organisms (10 org. m$^{-3}$). Numerical values above the box plots indicate the percent coefficient of variation of each data set.

the main ballast pipe is the best approach to capture variations due to the stratification of living organisms.

In summary, concentrations of organisms were significantly different among temporal segments of the drain, and in some cases, concentration estimates from a single drain segment were significantly different from the total drain concentration (i.e., the average of all drain segments). The occurrence of nonrandom vertical distributions of organisms has also been observed aboard ships,[16] and such heterogeneity was also evident in the TSS data collected in this study. Consequently, there does not appear to be an optimal time to sample during the discharge, and a single sample could lead to over- or underestimation of the total drain concentration, even when the sample is relatively large (i.e., ≥ 1 m$^3$). Smaller discrete samples would likely yield even more variation in organism concentration.

Thus, the sampling approach used is critical to obtaining an accurate population estimate when organisms are heterogeneously distributed in the ballast tanks (which will lead to heterogeneity throughout the tank drain). Indeed, this study and others indicate organisms are heterogeneously distributed in

ballast tanks, both in model tanks and those from ships at sea. Importantly, vessels may be deemed compliant to a discharge standard when discharging unacceptably high concentrations of potentially invasive organisms. Conversely, errors in sampling could lead to vessel fines for noncompliance when the vessel was, in fact, compliant.

While collecting complete continuous samples of a selected ballast tank(s) is possible, sampling an entire ship's discharge is not likely to be feasible aboard active vessels for routine compliance monitoring. The modeling results presented here suggest evenly spaced subsamples yield estimates of organisms with the lowest variation, but optimal sampling strategies will be refined as more data accumulate on the patterns of stratification within ballast tanks.

## ■ AUTHOR INFORMATION

**Corresponding Author**
*E-mail: matthew.first.ctr@nrl.navy.mil; phone: 305-293-4215; fax: 305-293-4213;

**Author Contributions**
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